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A new cave population of *Astyanax mexicanus* from Northern Sierra de El Abra, Tamaulipas, Mexico

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Abstract

The *Astyanax* genus represents an extraordinary example of phenotypic evolution, being their most extreme examples the blind and depigmented morphs, which have evolved from independent surface-dwelling lineages. Among cave organisms, *Astyanax* cavefish is a prominent model system to study regressive evolution. Before this study, 34 cave populations were known for the *Astyanax* genus to be inhabited by the cave morph. The majority of those cave populations are distributed in Northeast México, at the Sierra Madre Oriental (32 cavefish), in three main areas: Sierra de Guatemala, Sierra de El Abra, and Micos, and two in the Balsas basin in the state of Guerrero, Mexico. In the present study, we describe a new cave population found 4.5 km Southward of Pachón cave, the most northern cave population known for the Sierra de El Abra limestone. El Refugio cave is a resurgence with a mixed population of fish with different levels of troglomorphism, and surface fish, resembling other hybrid populations within the Sierra de El Abra. Based on a mitochondrial DNA characterization of the 16S ribosomal DNA sequence, we could identify the mitochondrial lineage of this population, which was placed closely related to the "New Lineage", sharing haplotypes with the surface (i.e. Arroyo Lagartos) and Pachón populations, instead of with the

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cave populations from Central Sierra de El Abra (e.g. Tinaja cave). El Refugio cave population gives additional evidence of the intricate history of this system, where migration, drift, and selection have shaped the evolution of the cave morphs through the independent episodes of the *Astyanax mexicanus* history.

Keywords
Astyanax, cavefish, regressive evolution, repeated evolution, Sierra de El Abra

Introduction

México represents a hotspot for troglobitic diversity in freshwater fish fauna, with seven different families with troglomorphic representatives including Characidae, Dinematichthyidae, Eleotridae Heptapteridae, Ictaluridae, Poeciliidae, and Synbranhidae families (Miller et al. 2005; Walsh and Chakrabarty 2016). This diversity of cavefish fauna is differentially distributed in the karstic regions of México, however, the Sierra Madre Oriental region, where cavefish populations of the *Astyanax* genus live (Characidae), includes a large number of cavefish populations, combined with a complex phylogeographic history, making this region a unique place to study regressive evolution.

Since the discovery of the first population of blind characins in 1936 in La Cueva Chica, in the southern portion of the Sierra del Abra limestone, by Salvador Coronado, the taxonomists of their time classified them as a new genus and species, *Anoptichthys jordani* (Hubbs & Innes, 1936), based on their troglobitic morphology (i.e., eye reduction and loss of body pigments). Shortly after, ichthyologists discovered another two cave populations of blind characids in the region. The first corresponds to El Pachón cave, *Anoptichthys antrobius* (Álvarez, 1946), and the second to *Anoptichthys hubbsi* (Álvarez, 1947), from Cueva de Los Sabinos, both at the Sierra de El Abra. At that time, each cave population was considered an independent episode of evolution from their surface ancestors and accordingly was described as different species. Afterward, Wilkens and Burns (1972), described the population of Micos, which was discovered in the 1960s, and characterized by fish that were not typically troglobitic, as they present similar pigmentation to surface fish, as well as a slight reduction in the vision. In terms of enzymatic diversity, they shown a clear differentiation from the previously studied troglobite populations (Avise and Selander 1972), and although Micos' diversity was not as high as in surface populations (being monomorphic for the majority of the loci), the authors shown that Micos cave presents the same alleles as the surface populations, suggesting that it is a recently adapted population, while others have suggested that it is a hybrid population (Mitchell et al. 1977). Thirty years later after their first discovery, during a multi-disciplinary exploration by Mitchell et al. (1977), cataloged 29 cave populations of *Astyanax mexicanus* (de Filippi, 1853) from Sierra de El Abra, Sierra de Guatemala, and Micos, in the states of San Luis Potosí and Tamaulipas in México. More recently, two new cavefish localities from the Sierra de El Abra, Chiquitita (Espinasa et al. 2018), and Toro #2 + La Calera system (Espinasa et al. 2020) were discovered. The recent discovery of new cave populations highlights the need to continue exploring this area, in order to better understand the extension and

connectivity that exists within the hydrological systems of the Sierra de El Abra, Sierra de Guatemala, and Micos areas.

Due to the extreme differences between the surface and cave *Astyanax mexicanus* morphs, the cave-dwelling morph has been considered a model organism to explore the genetic mechanisms underlying regressive evolution, particularly, vision loss, as well as the reduction in pigmentation (Protas et al. 2007; Gross et al. 2008; Protas et al. 2008; McGaugh et al. 2014; Jeffery et al. 2016). In recent years, the unprecedented development of molecular biology, developmental biology, and genomics, has allowed researchers to scan at the genome level the evolution of cave-adapted morphs in the Mexican tetra *Astyanax mexicanus*, not only in their phenotype, but also in their physiology, metabolisms, and behavior (McGaugh et al. 2014; Riddle et al. 2018; Ma et al. 2021; O'Gorman et al. 2021).

Regarding the evolutionary history of the cavefish populations of *Astyanax mexicanus*, multiple studies have shown evidence that cave populations have originated from two independent lineages (Strecker et al. 2004; Ornelas-García et al. 2008; Bradic et al. 2013; Herman et al. 2018), giving rise to parallel evolution of cave morphs within the species. In this regard, the cave populations from Sierra de El Abra area have been recognized as a different cave lineage, previously known as the old lineage, from the Micos and Sierra de Guatemala, previously known as the new lineage (Wilkes 1988; Gross 2012). Considering the hydrogeology of the region, five major drainages have been suggested (Espinasa and Espinasa 2016; Elliot 2018; Espinasa et al. 2020): 1) Sierra de Guatemala, draining into the Florida, Río Frío and Río Nacimiento springs, 2) Northern Sierra de El Abra, draining into Río Mante, 3) Los Sabinos in Sierra de El Abra, draining mostly into Río Choy, 4) Southern Sierra de El Abra, draining mainly to the Tampaon river, and 5) Micos, draining into Valles River.

With the development of genetic tools, it has been possible to characterize the evolutionary history and phylogeographic patterns of cave populations in northeastern Mexico. Among the first studies carried out with the mitochondrial DNA data, *Astyanax mexicanus* cavefish showed the presence of two different lineages that were named by Dowling et al. 2002 as lineage A and B (also known as the new and old lineages, see Gross 2012). The lineage A included the Northern and Southern extremes of the Sierra de El Abra (i.e. Pachón and Chica caves), together with the Sierra de Guatemala and Micos caves, grouped with most of the local surface populations. While the caves with the haplotype B included the Yerbaniz and Los Sabinos area *sensu* Elliott (2016) in the central Sierra de El Abra, (Dowling et al. 2002; Strecker et al. 2004; Ornelas-García et al. 2008; Bradic et al. 2012; Espinasa et al. 2020). Based on genomic data, we now know that the extremes of the Sierra de El Abra (i.e. Pachón and Chica) belong to the same lineage that the rest of the Sierra de El Abra caves, but they have undergone a mitochondrial introgression with surface fish (Bradic et al. 2013; Herman et al. 2018; Moran et al. 2022). Moreover, the Sierra de El Abra corresponds to an independent lineage to the Sierra de Guatemala and Micos corresponding to independent events of cave adaptation (Bradic et al. 2012; Strecker et al. 2012; Herman et al. 2018). In addition, we know that in some populations from the Sierra de El Abra, Micos, and Sierra de Guatemala, there has been gene flow with surface populations and this contact has been differential between the populations of these areas (Bradic et al. 2012; Herman et al. 2018; Moran et al. 2022). Thus, the complete interfertility between surface

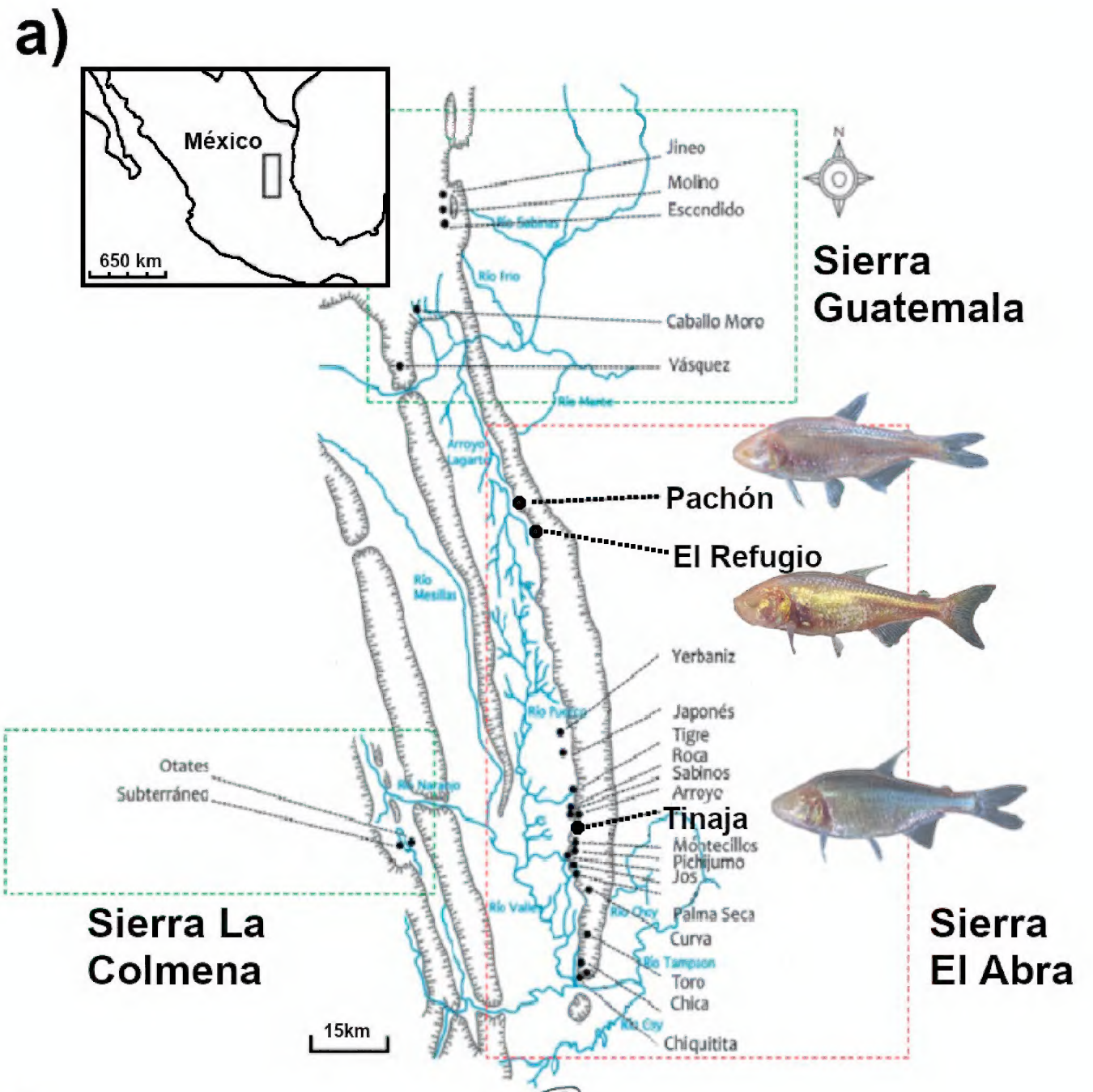
and cave populations has allowed the exploration of the genetic architecture underlying phenotypic changes in the cave-adapted morphs (Hubbs and Innes 1936; Alvarez 1946, 1947; Sadoglu 1956; Wilkens and Hüppop 1986; Wilkens 1988). Finally, based on coalescence methods, and important genomic representation, it has been possible to support the hypothesis proposed by Avise and Selander (1972), that the origin of cave populations is very recent (<200,000 years, Fumey et al. 2018; Herman et al. 2018), contrary to what had been suggested with mitochondrial molecular clocks (Strecker et al. 2004; Ornelas-García et al. 2008).

In the present study, a new population is described for the northern region of Sierra del Abra. A general description of its geohydrology is included. In addition, a brief description of the phenotypic variation found in the cave, and the characterization of a mitochondrial ribosomal marker (r16S) were included. Based on this information, it is concluded that this population could correspond to a mixed population, with a large phenotypic diversity in terms of the degree of regression of vision and pigmentation traits, as has been reported in other hybrid caves such as Chica. Regarding the geometric morphometric variation, we recovered El Refugio populations as intermediate between the Pachón and Tinaja caves, giving evidence about the common ancestry between the caves within the Sierra de El Abra system, despite the large introgression between the Río Lagartos surface population and El Refugio cave.

Methods

The "El Refugio" cave (22°34'28.50"N, 99°01'30.74"W, 231 masl), takes its name from the closest town in the state of Tamaulipas, México. The cave was discovered by Jean Louis Lacaille in April 2020, and troglomorphic fish were seen in some of the cave pools (Figs 1, 2). A second expedition trip was organized on February 23th, 2022 with the purpose of fully documenting and collecting specimens of this cave. On that day, a spring only 100 m from the Refugio cave (22°34'25.50"N, 99°01'29.61"W) was also visited. The Manuel de la Fuente spring is a 3 m deep open-air well from which the locals extract water for consumption using a pipe (Fig. 3). The well harbors an electric pump. Upon inspection, two cavefish were seen swimming under the sunlight. A cable ladder was used to descend the well and collect both fish with a hand net. Underwater conduits were evident at the bottom of the well from which fish could swim out, but they are probably too small for human exploration with scuba equipment.

To explore the El Refugio cave, a cable ladder was also employed to access its 2 m entrance pit. The cave was surveyed using a DistoX2 device, and the TopoDroid app on a tablet. Survey data were processed using Visual Topo ver. 5.17, and drawn using Adobe Illustrator. To capture specimens, hand-held nets were used. Photographs were taken in the field with the help of a small glass tank and an iPhone 11 camera. A small fin clip was taken with scissors for DNA studies from all specimens. Voucher samples were stored at the Colección Nacional de Peces, IBUNAM, Mexico.



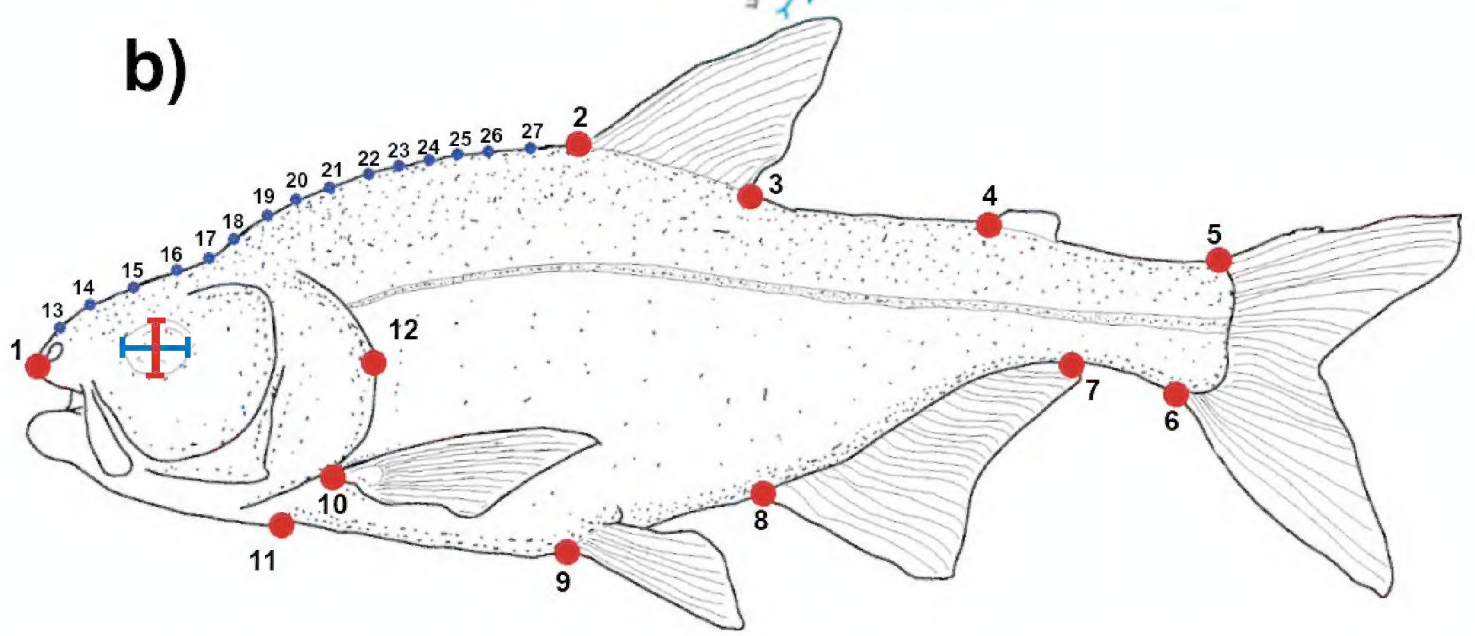


Figure 1. a simplified map edited from Elliott (2016), with the new cavefish locality of El Refugio, as well as the localities included in the morphological characterization: Pachón and Tinaja in Sierra de El Abra system **b** position of the 12 homologous landmarks (red) and the 15 pseudolandmarks (blue) curve for geometric morphometric analysis. Within the eye orbit the vertical line (red line), corresponds to the Orbital Diameter Height, and the horizontal line (blue line) corresponds to the Orbital Diameter Length.

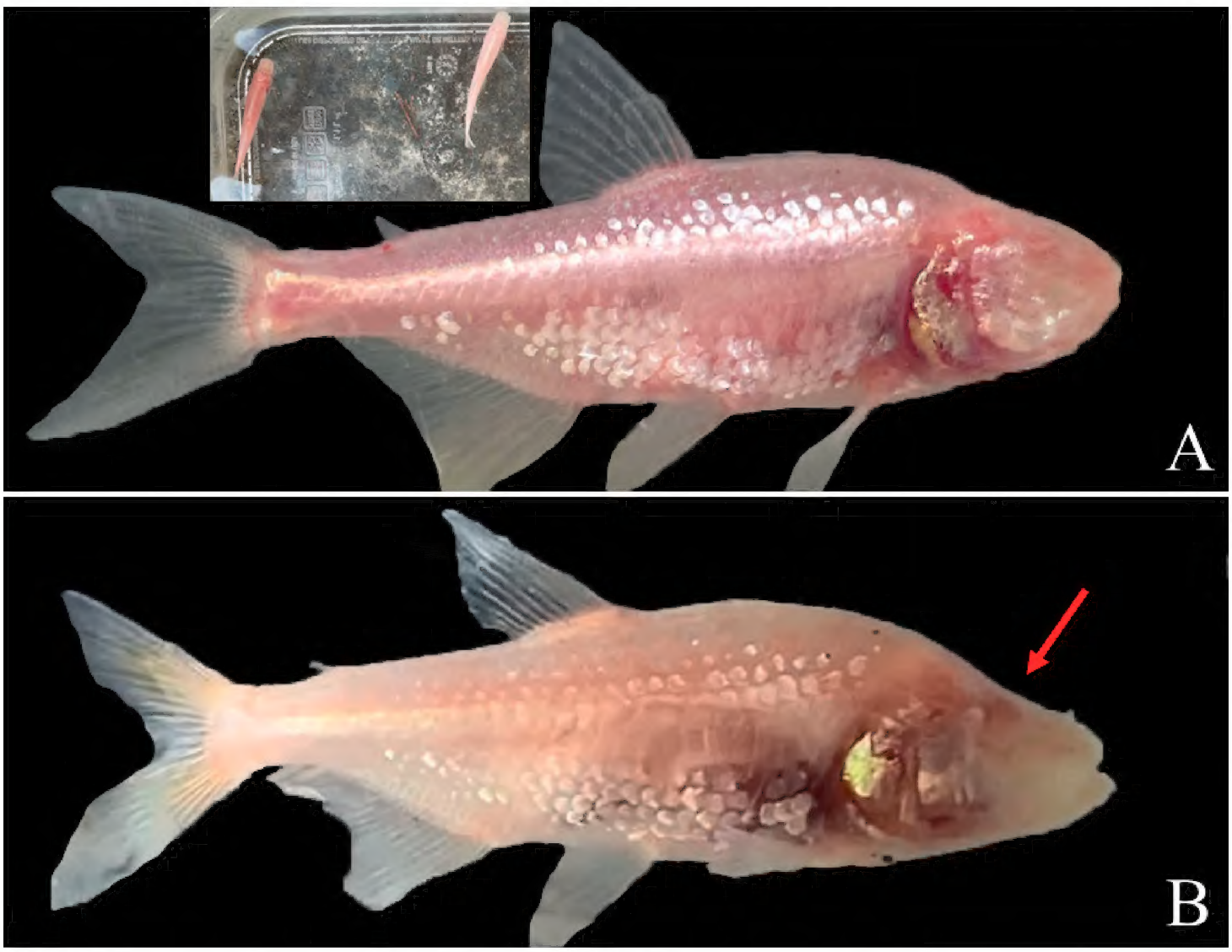


Figure 2. Highly troglomorphic fish are found in the El Refugio cave and in the nearby well. The two fish in the insert were found in the well-used by locals to extract water. In this well, troglomorphic fish are seen swimming under the sunlight. The most troglomorphic fish in this cave system have two types of colorations, pink (**A**) and white (**B**). These two colorations are shown also in the specimens in the insert. Notice also that eye regression and skull modification create a downward hump in some specimens (red arrow).

For the collection of cave specimens, permission was obtained from the competent Mexican authorities (SEMARNAT SGPA/DGVS/03334/22). All of the fish collected were kept alive in the laboratory of Patricia Ornelas-García, IBUNAM, for breeding in captivity, which can serve as a stock for future studies.

Genomic DNA samples were obtained following standard methods for DNA purification using Qiagen's DNeasy Tissue Kit by digesting a fin clip of the individual in the lysis buffer. A fragment of the mitochondrial 16S rRNA was sequenced for 6 troglomorphic, one hybrid, and one epigean fish, all collected inside the El Refugio cave. The 16S rRNA fragment was amplified and sequenced using the 16Sar (CGCCTGTTTATCAAAAACAT) and 16Sb (CTCCGGTTTGAACTCAGATCA) primer pair for 16S rRNA. Amplification was carried out in a 50 µl volume reaction, with QIAGEN Multiplex PCR Kit. The PCR program consisted of an initial denaturing step at 94 °C for 60 sec, 35 amplification cycles (94 °C for 15 sec, 49 °C for 15 sec, 72 °C for 15 sec), and a final extension step at 72 °C for 6 min in a GeneAmp PCR

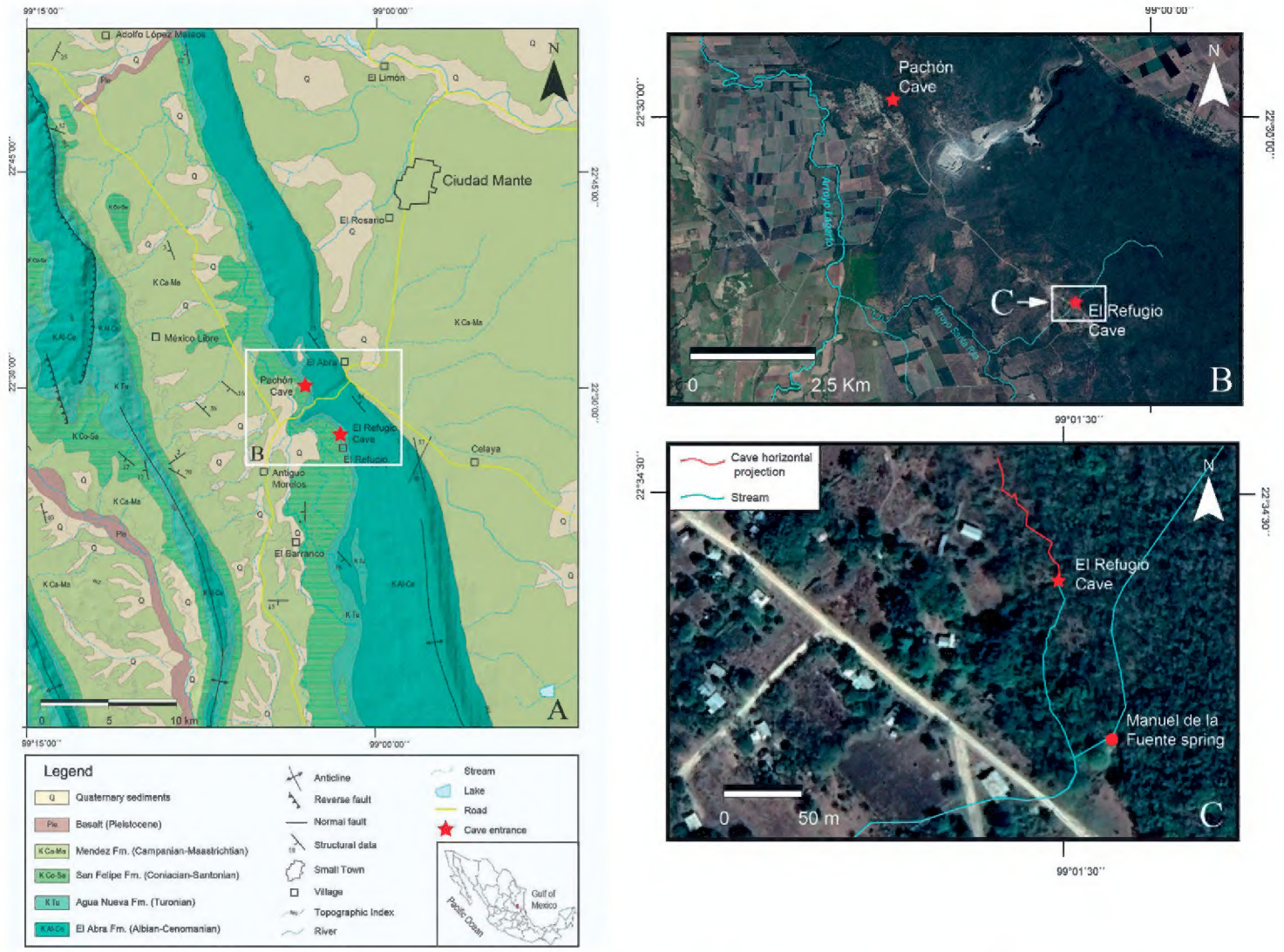


Figure 3. A general geologic map of the northern part of the Sierra del Abra showing the locations of the El Refugio and Pachón caves entrances at the geological contact between Agua Nueva and San Felipe formations (modified from Carta Geológico-Minera Ciudad Mante F14–5) **B** satellite view showing the principal streams near the cave entrances **C** detailed view of B. The cave horizontal projection of the El Refugio cave is observed in a red line as also the position of the Manuel de la Fuente Spring. In blue are stream beds that only carry water during the rainy season.

System 9700 (Perkin Elmer). PCR amplified samples were purified with the QIAquick PCR purification kit and directly sequenced by Elim Biopharm Genomic Services (https://www.elimbio.com/services/dna-sequencing/). Chromatograms obtained from the automated sequencer were read and contigs were made using the sequence editing software SequencherTM 3.0. External primers were excluded from the analyses. BLAST was used to identify GenBank sequences that resemble the specimens. Sequences were aligned with ClustalW2.

For comparison, DNA sequences were aligned to sequences from localities reported previously by Espinasa et al. (2020). We included the mitochondrial haplotypes classified as lineage A or B, which for lineage A the following populations are included: Comandante surface river (affluent of Río Frío), Sótano del Molino, Sótano del Caballo Moro, Cueva del Pachón, Toro #2, Cueva Chica, and Cueva Chiquitita. Mitochondrial lineage B included samples from the Rascón surface stream, Tamasopo surface stream, Cueva de Los Sabinos, Cueva de la Tinaja, and Cueva de la Curva.

Morphological characterization

To characterize the morphospace of the El Refugio population and their relationship with other populations from the Sierra de El Abra, we performed landmark-based Geometric Morphometrics (GM) analysis on the lateral left-hand view of the body shape. We digitized 12 homologous landmarks and a curve with 15 pseudo landmarks with the TPSDig2 v. 2.31 (Rohlf 2015; Fig. 1), of 45 organisms, 19 from El Regio (8 completely troglomorphic, and 1 apparently from the surface, and 10 with intermediate characteristics), 10 from Pachón, and 10 from Tinaja, and 6 from a surface population (from Río Florido, Pánuco basin). A Generalized Procrustes Analysis (GPA) was performed with the "gpagen" in Geomorph v. 4.02 (R Core Team 2019), and with the 'curve' argument the sliders were defined with the Procrustes distance criterion to optimize the position of the sliding reference point during the GPA.

To avoid a size effect by allometry, the residuals of the regression of the shape on the centroid size (CS) were calculated with the function "procD.lm". Once the residuals were obtained, an allometry-free shape was created, using these residuals of the morphological data. The analyses were made on this allometry-free shape. To assess the variation in body shape, we used principal component analysis using the "gm.prcomp" function. This is an ordering analysis that captures the multidimensional variation that is inherent in body shape (Zelditch et al. 2004). Deformation grids were used to describe the morphospace at the ends of the axes, which allowed us to observe the difference in the relative shape associated with the components that explain a greater percentage of variance (Zelditch et al. 2004).

In addition, discriminant analysis of principal components (DACP) (Jombart et al. 2010; Jombart et al. 2011) was performed in RStudio (RStudio, Team 2020). DAPC is a multivariate analysis designed to identify and describe clusters of morphologically related individuals. DAPC relies on data transformation using PCA as a prior step to Discriminant Analysis (DA), ensuring that variables submitted to DA are uncorrelated. DA method defines a model in which genetic variation is partitioned into a between-group and a within-group component and yields synthetic variables that maximize the first while minimizing the second (Jombart et al. 2010). DAPC was performed using the geometric morphometric data comparing the individual assignment considering the "find.clusters" function. We run a K-means clustering algorithm (which relies on the same model as DA) with different numbers of clusters, each of which gives rise to a statistical model and an associated likelihood. With the "find.clusters" function, we evaluate from K = 1 to K = 6 possible clustering in 10 different iterations (DAPC) (Jombart et al. 2011). The selection of the number of principal components was carried out with a cross-validation analysis. The clusters or conglomerates resulting from the DAPC were visualized in a scatter diagram, using the first two discriminant functions, representing individuals as points. A variance analysis was carried out, using the population as a factor (i.e., El Refugio, Pachón, and Tinaja), and the Centroid size as a covariable, as well as the interaction between population and CS, was estimated in R ver. 3.6.1 (R Core Team 2019).

An Orbital Diameter Height was estimated (red line in the orbit, Fig. 1b), and the Orbital Diameter Length (blue line in the orbit, Fig. 1b), both measures were corrected by the standard length of the fish, and a boxplot was carried out to graphically compare the median and standard deviation among Pachón, El Refugio and Tinaja cave populations, in contrast with a surface population (i.e., Río Florido).

Results

El Refugio cave is part of a series of resurgences (i.e., point at which an underground stream reaches the surface and becomes a surface stream) in the area that include the nearby well and Refugio cave itself. The cave shows tectonic control, and the orientation of the galleries follows the main NW-SE fault (Fig. 3A). Cavefish were encountered at two close localities: 1) El Refugio cave (Fig. 3B), and 2) at the Manuel de la Fuente spring (Fig. 3C). The two localities are aligned to the fault direction and separated only 100 m from each other, suggesting that they are hydrologically connected and allowing the cavefish to move from one to the other.

Regarding surface drainage, during the dry season water is pumped out through the well, but during the rainy season, high water volumes flow from both localities. During the rainy season, water from the Manuel de la Fuente spring and El Refugio cave resurgence join forming a creek that flows to the Arroyo Santa Rita downstream and is captured by Arroyo Lagartos (Fig. 3B, C).

Cave description

The El Refugio cave has a 2 m pit entrance that leads to a single gallery that develops parallel to a local fault going northwest with an average direction of 330°. The cave's total length is 98 m and has a 4 m total depth. The cave ceilings are low, and therefore requires crawling most of the time. El Refugio is a shallow cave that acts as a resurgence in the wet season, where water floods the entire gallery and flows out from the cave entrance. El Refugio cave has a few small pools the cave forms of *Astyanax mexicanus* as well as surface and intermediate fishes (Fig. 4).

During the dry season when El Refugio cave was explored, dry galleries were interspersed with some pools. The first was a very shallow puddle under a small bat colony (*Astyanax* pool 1 in Fig. 4). Only a surface fish and a troglomorphic fish were spotted. The second pool is a crevasse about 1 m long (Fig. 5A), but several meters deep, hosting tens of fish. A deep crevasse appears to join further underwater passages (Fig. 5B). Many different cavefish were seen going into and out of this void, suggesting a large underwater conduit inaccessible to humans, perhaps joining the aquifer (*Astyanax* pool 2 in Fig. 4). The third pool was the longest, about 10 m long, and was inhabited by both *Astyanax mexicanus* cavefish and mysid cave shrimp *Spelaeomysis quinterensis*. Low ceilings make you crawl inside the water, although an air current hints that the cave has furthermore unexplored passages (*Astyanax* pool 3 in Fig. 4).

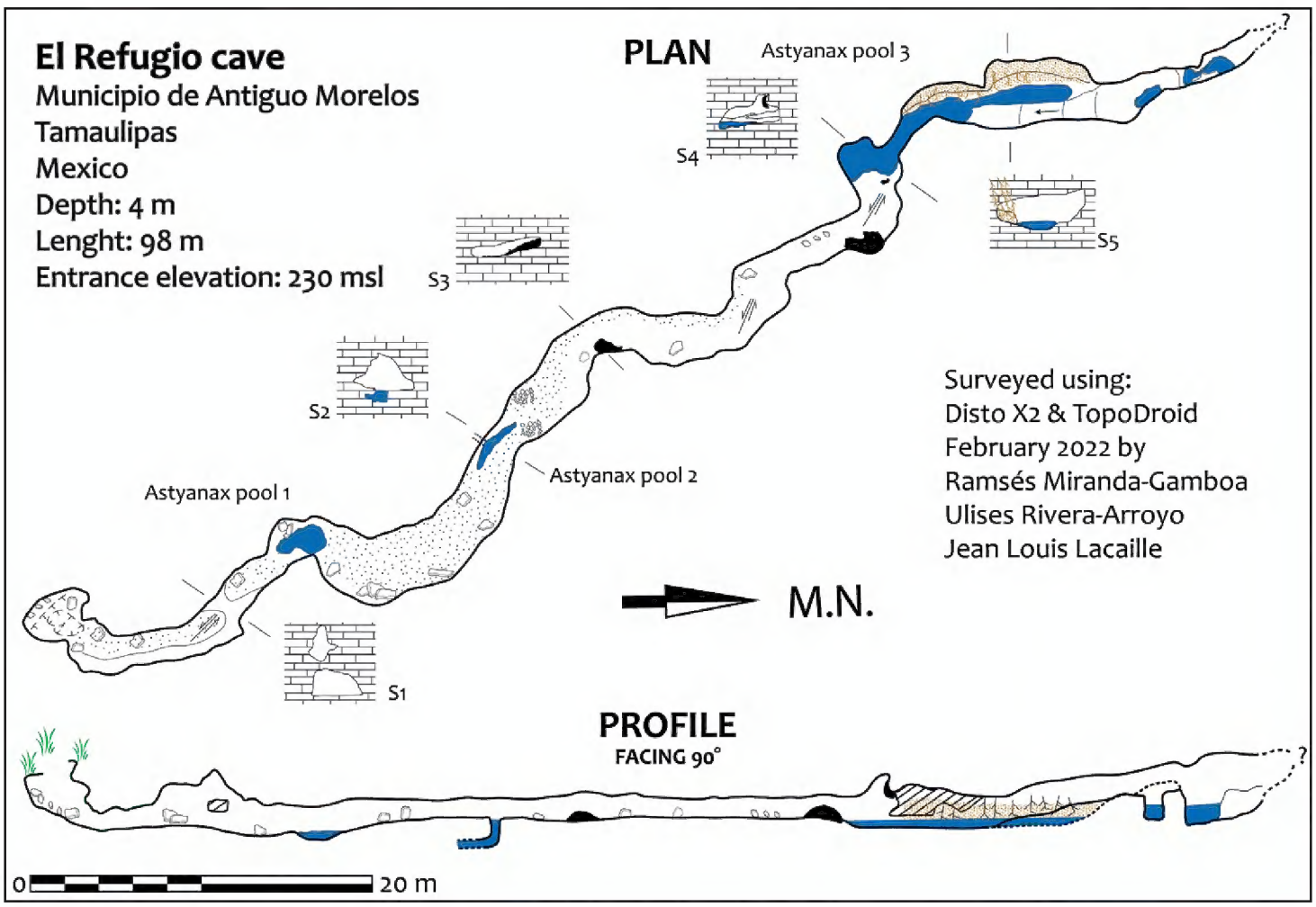


Figure 4. Map of El Refugio cave.

The initial part of the cave has a bed of sediments and guano from hematophagous bats that shelter in the cave. On the left wall of the third pool, there is a long mound of mud attached to roots coming from the ceiling or the walls, indicating a near connection to the surface (Fig. 4). In the final part, there is a little slope reaching a couple of deeper pools without cavefish. In the second one an apnea is required to get to the last chamber. In this part, the ceiling is high enough to stand straight. The cave ends with a small crack impenetrable for humans but seems several meters long (Fig. 4, dashed line at the farthest part of the cave in the profile and plan).

El Refugio is an active cave that discharges water in the rainy season and as such, inside the cave, there is evidence of this. All the Refugio walls are covered with scallops showing the dynamic behavior of water (Fig. 5B). There are plenty of dissolution domes in the ceiling. They have different sizes, most of them are about 15 cm long, as can be seen in Fig. 5C, but a couple of them are about meter-sized (Figs 4, 5D) and are located principally just over fissures allowing the possibility of water to seep up and dissolve the rock.

As it is possible to observe in the cross sections of the El Refugio cave, has the characteristic shape of a phreatic conduct whose shape is elliptical (Fig. 4), and develops along the fissure axis as can be seen in Fig. 5E, F. This suggests a slow flow of floodwaters through the cave. Furthermore, there is another evidence of what possibly is a local water table. Many walls of the cave have calcite adhered to them, as is shown

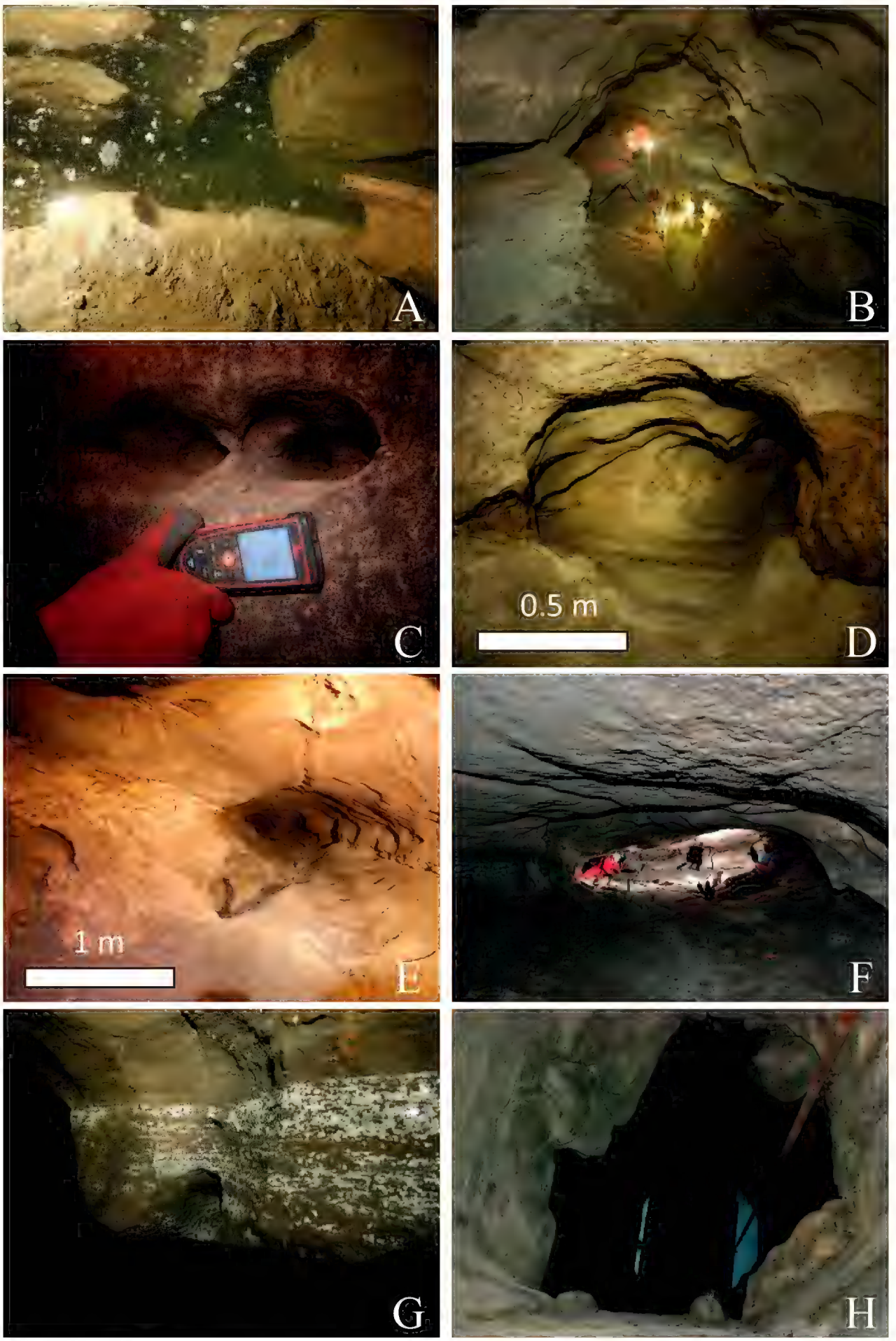


Figure 5. Some important features of the cave **A** an underwater crevasse about 1 m long in the second pool **B** aforementioned crevasse on the floor leading to underwater passages **C, D** dissolution domes of different sizes along the cave galleries, suggesting that during rainy season, the whole cave is under water **E, F** examples of phreatic conducts with a typical elliptic morphology developed along fissures **G** calcite crystals covering the walls cave as evidence of multiple variations in the water table **H** view from the surface of the Manuel de la Fuente well.

in Fig. 5G, indicating that the water level remained in the same position for a long time. Additionally, there is a testimony of local people who actually saw in May of 2021 that the water level was higher than the one documented in February of 2022. Finally, the well (Fig. 5H) which also hosts blind fishes and probably is connected to the El Refugio cave, is a year-round source of drinking water for the people of the El Refugio community and its level doesn't decrease over time. All these are evidence that El Refugio cave is related to a water table or aquifer.

Specimens

This cave hosts a mixed population; thus, we can find troglobitic (with complete loss of vision and pigment), surface and intermediate morphs. From the cave survey of the specimens that were observed in detail, 31.8% (N = 7) were highly troglomorphic specimens, with no pigment or external remnants of eyes, 54.5% (N = 12) were intermediate with either some type of eye and/or pigment, and 13.6% (N = 3) were surface-like fish. It is likely that surface fish are more abundant within the population, but since they react to light, they are more difficult to catch and swam away. The troglomorphic fish had either characteristic pinkish or white colorations (Fig. 2). The surface-like fish were fully pigmented, with large eyes, and as mentioned, responsive to light. It is the authors' assumption that they are simply surface fish that during the rainy season swam inside. While during the dry season there is not a surface stream, it is expected that when flowing in the rainy season, the temporal stream gives easy access to surface fish from the nearby Arroyo el Lagarto. Introgression between the surface morph and the cave morph is suggested by the presence of individuals with a large phenotypic variation in the eye-regression level, from entirely absent eyes, to embedded eye cist, embedded small eye, a small eye with closed pupil, a small eye with open pupil, to large eyes (Fig. 6A–F). Similarly, we could observe a wide variation with respect to the combination between body pigments and eye regression levels, that is small-eye or eyelss without pigment, eyed and eyeless with a yellow pigment, eyeless with a back caudal spot and eyed fish with black spot and yellow pigments at the caudal fin (Fig. 6G–L).

Mitochondrial DNA

A 16S rRNA fragment of 572 bp was sequenced for six troglomorphic fish, one hybrid, and one epigean fish from El Refugio cave. All specimens analyzed have the haplotype A (GenBank# AP011982.1), identical to the Pachón cave sequence and to the local surface population (i.e., Arroyo Lagartos). When comparing El Refugio sequences to the mitochondrial haplotype B from Rascón and Tamasopo surface streams fish, El Refugio specimens differed by 2–3 bp. When we compared El Refugio sequences with Sabinos, Tinaja, and Curva caves sequences, they differed by 5 bp. Thus, our 16S rRNA sequences recovered El Refugio cave specimens closer to the northern Sierra de El Abra region, instead of the Central part of the Sierra de El Abra (Suppl. material 1).

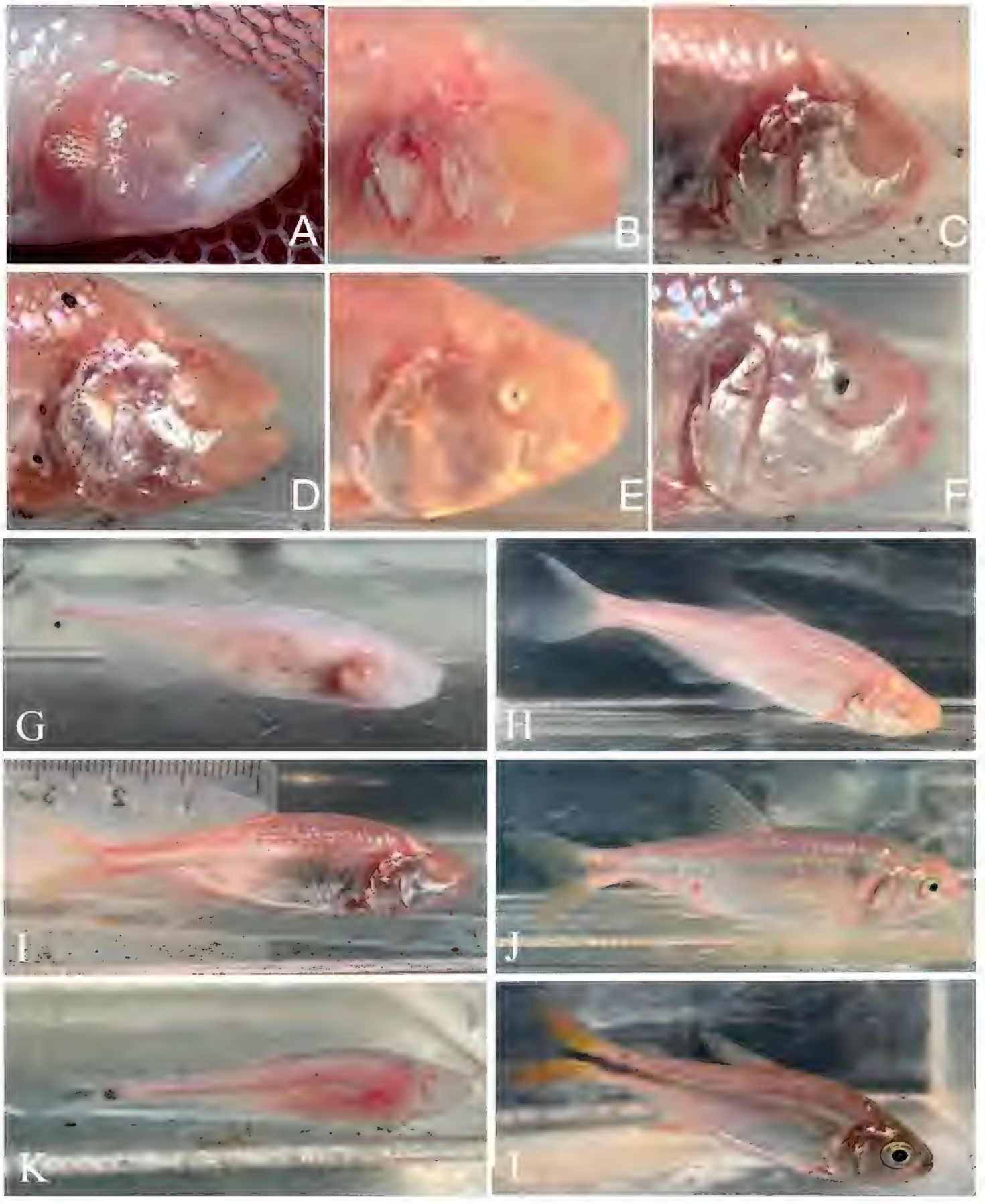


Figure 6. Eye regression variations within the El Refugio population. In the figure, we can observe from entirely absent eyes (**A–C**), to different levels of vision regression (**D–F**). Regarding the body and eye regression combined we also observed a wide variation **G, H** eyeless and small-eye fish without pigment **I, J** eyeless and eyed fish with yellow pigment, and eyeless fish with a caudal black spot (**K**) **L** eyed and black spot and yellow pigment in the caudal fin.

Morphological variation

The first two components explain 43.67% of the cumulative variance (Fig. 7A, Suppl. material 2). PC1, which explains 25.84% of the variance, describes the variation in

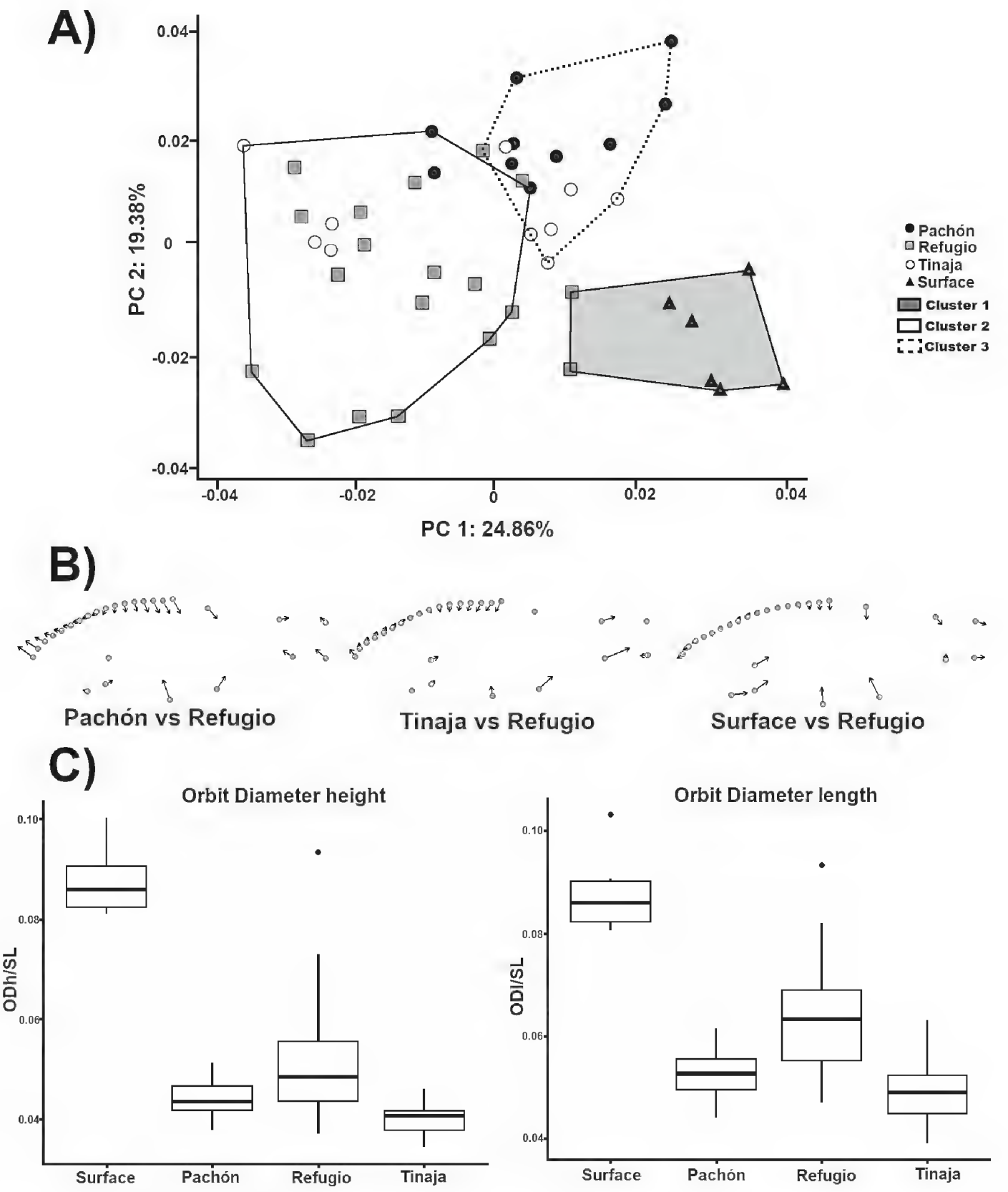


Figure 7. A PCA of the body shape of the three cavefish populations. At the end of each axis, the shape obtained by the positive and negative ends was placed. The hullboxes delimit the clusters obtained through K-means analysis. Black hullbox = cluster 1 (surface morph), Filled hullbox = cluster 2 (intermediate individuals), dotted line hullbox = cluster 3 (the most troglomorphic individuals) **B** comparison of the consensus shape of the El Refugio population (deformation vectors) vs. the consensus shapes of Pachón, Tinaja, and Surface (points) **C** boxplot of the orbital diameter corrected by the SL for the Surface, Pachón, El Refugio and Tinaja populations.

body height, with slender bodies at the negative side of this component, and deeper bodies at the positive side. The PC2 explains 17.83% of the variance, and we observed differences in the dorsal profile of the head, discriminating between troglobitic and surface individuals, thus, at the most positive side of PC2 we recovered the Pachón, Tinaja

and El Refugio individuals with a concave head profile (i.e., duck-bill-like rostral flattening), and at the negative side of the PC2, we recovered the individuals with a softer and streamlined dorsal profile of the head, characteristic in surface-like fish (Fig. 7A).

For the DAPC, a total of 38 PC were used, and based on the Silhouette method we recovered K = 3 as the optimal number of clusters (Fig. 7A). According to this result, the first cluster (black hullbox) gathered the surface population (i.e., Río Florido), with two surface-like individuals from El Refugio. The second cluster (filled hullbox) with the largest morpho-space considering both PC axes, grouped most of the fish from El Refugio population, with intermediate morphs between the surface and the cave individuals. Although in this cluster we find Pachón and Tinaja fish, both are distributed on the positive side of PC2, with a concave dorsal profile of the head, while the El Refugio fish specimens were characterized by a slender body, with a broad distribution in the second axis (PC2), corresponding to a gradient in the degree of dorsal flattening of the head. Finally, we have the third cluster (dotted line hullbox), which included fish from Pachón, Tinaja and two individuals from El Refugio, which correspond to the most troglobitic fish.

The MANCOVA support differences among populations, but not by the centroid size or the interaction between CS and the populations (Table 1). These differences among populations can be appreciated in the comparisons among the consensus forms obtained for each population (Fig. 7B). In both comparisons, we use the Pachón and Tinaja cave populations as the reference (the gray points), while El Refugio was depicted by the deformation vectors. In general, the differences for El Refugio population were similar with respect to the other two populations, thus, it has a slender body in contrast to the other two cave populations, and additionally, Pachón cave presents a stronger duck-bill-like rostral flattening. In Tinaja the caudal peduncle was larger than the other two populations (Fig. 7B).

Finally, in the orbital diameter bloxplot (Fig. 7C, Suppl. material 2), was possible to observe a wide variation in the El Refugio population, corresponding to a wide spectrum of phenotypes, from the most troglomorphic with a completely absent eye to the individuals with a surface-like eye, giving additional evidence of the mixed nature of this population. In Table 2 we present the average values for the orbital diameter measurements and their proportions with respect to the standard and the head length. For El Refugio population, the average orbital diameter height (OD_H) is 2.35 mm (SD = 0.77 mm), and the average orbital diameter length (OD_L) is 2.88 mm (SD = 0.72 mm). While the average values were lower in both Pachón and Tinaja caves. In Pachón we recovered an OD_H = 2.28 mm (s.d. 0.24 mm), and OD_L = 2.71 (s.d. 0.24 mm). For Tinaja de OD_H = 2.35 mm (s.d. 0.35 mm), and

Table 1. MANCOVA made with GM body shape data. The asterisk is for statistically significant p-values.

	DF	Pillai	F	p
Population	3	1.97	17.12	> 0.001*
Centroid Size	1	0.13	1.28	0.249
Pop*CZ	3	0.42	1.48	0.139
Residuals	37			

Table 2. Average of the Orbit diameter height (OD_H) and length (OD_L), and their respective proportions with respect to the fish's Standard Length (SL) and Head Length (HL). All the measurements are presented in mm. The standard deviation is presented for each measurement.

	Refugio	S.D.	Pachón	S.D.	Tinaja	S.D.	Florido river	S.D.
SL	45.42	9.61	51.83	1.93	58.79	7.98	58.30	8.20
OD_H	2.35	0.77	2.28	0.24	2.35	0.35	5.11	0.81
OD_L	2.87	0.73	2.71	0.24	2.88	0.39	5.13	0.86
HeadL	11.90	2.32	12.62	0.68	14.02	1.57	13.72	2.29
OD_H/SL	0.05	0.01	0.04	0.00	0.04	0.00	0.09	0.01
OD_L/SL	0.06	0.01	0.05	0.01	0.05	0.01	0.09	0.01
HeadL/SL	0.26	0.02	0.24	0.02	0.24	0.01	0.23	0.01
OD_H/HL	0.20	0.06	0.18	0.02	0.17	0.02	0.37	0.03
OD_L/HL	0.24	0.05	0.22	0.02	0.21	0.02	0.38	0.03

OD_L = 2.88 mm (0.39 mm). As expected, the orbit diameter values were higher in the surface population (Table 2).

The individual dot at the surface boxplot corresponded to the biggest fish (SL = 68.2 mm, with an OD_H = 5.53 mm, and OD_L = 5.5 mm), while for the El Refugio the individual dot corresponds to a surface-like fish, with completely functional eyes (SL = 70.6 mm, with an OD_H = 4.24 mm, and OD_L = 4.49 mm, Suppl. material 2).

Discussion

Since the discovery of the first cave population of *Astyanax mexicanus* in 1936 at La Cueva Chica, the history of cave explorations in the Sierra de El Abra has been extensive, with an important increase in the number of caves discovered during the 70's, as described in Mitchell et al. (1977) and Elliott (2018). In recent years (Espinasa et al. 2018; Espinasa et al. 2020), new cave populations have been discovered within southern Sierra de El Abra system, which has increased our understanding of its limits and connectivity between cave and surface populations.

The present study is an example of this, where the discovery of the El Refugio cave, has increased the number of populations in the northern part of Sierra de El Abra *sensu* Elliott (2018). Before this study, only Pachon and Venadito caves were known to harbor troglomorphic fish. The distance between El Refugio cave and Arroyo Santa Rita is 1.7 km, and the vertical height is 40 m, which makes it possible for surface fish from Arroyo Lagarto to swim upstream (rheotaxis) and reach the cave's entrance. The distance between El Refugio cave and Pachón cave is 4.5 km.

Some of the caves in the Sierra de El Abra, Sierra de Guatemala, and Micos mountain ranges capture surface streams, so these caves play the role of active sinkholes in the karst landscape of these regions. Nevertheless, Pachón and El Refugio caves behave differently, that is, they are resurgences where water flows out of the cave, joining the surface streams. For the Pachón cave, it has been hypothesized that the main connection with the surface drainage is given only in heavy rainy seasons when the cave's

water overflows present as a resurgence and the subterranean water achieves surface streams creating a sporadic path for surface fish to get into the caves (Bradic et al. 2012; Espinasa and Espinasa 2016).

Previous studies have suggested a temporal connection between the Pachón cave and Arroyo Lagartos (Bradic et al. 2012), from the former to the latter. Thus, during wet years, the Pachón entrance is a resurgence, creating a path for connecting surface fishes from Arroyo Lagartos. The nearest branch of Arroyo Lagarto is only 1 km away, but there is a 50 m vertical distance between the stream and the cave's entrance. In this regard, in 1991 some individuals with variable eye sizes and melanin pigmentation were observed in Pachón cave (Langecker et al. 1991), suggesting surface fish gained access to the cave. In El Refugio cave, we observed a mixed population, with a great variation in the pigment and eye loss, from the completely pigmented and eyed fish to the albino with complete eye loss, suggesting that this population maintains a much higher gene flow with the Arroyo Lagartos surface population than Pachón cave does.

In this regard, the Sierra de El Abra formation corresponds with a Cretaceous marine rock deposited in the platform, basin paleoenvironments (Agua Nueva, San Felipe, and Mendez formations), and Quaternary igneous rocks and alluvial deposits (Elliott 2018). The oldest unit cropping out in the area is the Sierra de El Abra formation, which is a succession of reef limestones and dolomites deposited during the Albian-Cenomanian system (Aguayo-Camargo 1975). The Agua Nueva Formation is composed of interbedded carbonaceous shale and limestone rocks of the Turonian age (Carrillo-Bravo 1971). The San Felipe formation comprises an alternation of limestones, marlstones, and tuffs. The age of this unit has been determined through paleontological studies as Coniacian-Santonian (Muir 1936; López-Ramos 1979). The Mendez formation has been described as a Late Cretaceous (Campanian-Maastrichtian) succession of shale, marlstone, and scarce sandstone and limestone (Carrillo-Bravo 1971; Keller et al. 1997).

The history of this area began in the Late Cretaceous and extends to the Eocene (Fitz et al. 2018), this deformational event creates a typical anticline-synclinal structural arrangement that dominates de geomorphology of the region. Several caves in the area, including the El Refugio and Pachón caves, were developed near the contact between the Agua Nueva and San Felipe formations. This geological contact probably acts as an inception horizon (Filipponi et al. 2009) (Fig. 3A, B). The karstification develops through the Agua Nueva formation and then it is limited due to the lower permeability of the San Felipe formation rocks.

The probability that a surface fish can get into these two caves (i.e. Pachón and El Refugio) is different since for the Pachón cave the entrance is at an altitude of 203 masl and corresponds to a perched system on a steep hill (Suppl. material 3: fig. A), being the base level of the valley at 175 masl, thus, corresponding with a geographic barrier that can only be avoided during important floodings. On the contrary, the El Refugio cave entrance is at 229 masl, and at the same base level as the temporal surface stream (Río Lagartos), thus, it is likely that every rainy season surface fish have direct and easy access to El Refugio cave.

By other hand, the subterranean connectivity between these two caves nowadays seems unlikely, since, between Pachón cave and El Refugio cave, there is a fossil canyon

of 100 m deep (Suppl. material 3: fig. B), which can act as a barrier. Espinasa and Espinasa (2016) argued that as tectonism uplifted the El Abra region, the limestone eroded, changing the course of the rivers. Around one to four million years ago, the Río Comandante, which ran through the aforementioned canyon, changed its course north, to its present position in La Servilleta canyon. There are several of these fossil canyons in the Sierra de El Abra and they have been shown to be considerable biogeographical barriers to the dispersal of aquatic organisms (Espinasa et al. 2020). Further studies evaluating the gene flow between these two cave systems can give additional information about their present and historical connections.

The geometric morphometric analyses showed very interesting results, with some individuals from El Refugio cave being clustered with Tinaja cave, while two individuals analyzed from the El Refugio fish were assigned to the Pachón cave cluster. Based on the mitochondrial data, we recovered haplotype A for the El Refugio cave, which is the same haplotype found in Pachón cave, and different from Tinaja cave. Previous studies have shown the Pachón cavefish exhibit a disparity between nuclear and mitochondrial data, with the nuclear data clustered with the rest of the populations from the Sierra de El Abra, while the mitochondrial haplotypes clustered with the nearby surface fish (Dowling et al. 2002; Strecker et al. 2004, 2012; Herman et al. 2018). The presence of the same haplotype between surface and Pachón cavefish has been suggested as the result of a recent episode of introgression with surface fish (Langecker et al. 1991). In 1986, individuals with variable eye sizes and melanin pigmentation were observed in the former albino and eye-reduced Pachón population (Langecker et al. 1991). We suggest a mitochondrial introgression from the surface population to El Refugio cave, rather than shared ancestry (i.e., haplotype A), similarly to the patterns observed at Pachón cave (Dowling et al. 2002; Strecker et al. 2004, 2012; Herman et al. 2018), however, further studies considering the nuclear information are need to better understand the historical and recent connections among northern caves of the Sierra de El Abra (i.e., Pachón and El Refugio), and their relation with Los Sabinos area. Finally, it is important to highlight that this new cave population is highly vulnerable since there are important impacts and threats to this new population that are mainly human-derived. Principally, the well in the El Refugio cave system has a pipe from which water is extracted for local consumption, which potentially can lead to habitat destruction since it is not clear the extraction volume by this pipe. Another concern is that the cave is located within an urban settlement, which can affect the water quality of the wastewater discharged into the local aquifer. Finally, El Refugio cave gives access to only small pools with very small populations of at most tens of individuals. Thus, it is important to prevent overfishing by scientists or common people.

Conclusions

The Sierra de El Abra corresponds to a unique system in which we can find more than 200 caves, holding a great diversity of fauna (Mitchell et al. 1977). The *Astyanax mexicanus* cavefish have adapted to these cave environments, occupying 33 caves in the El Abra

region (Elliott 2016; Espinasa et al. 2021). El Refugio cave is a newly described resurgence cave inhabited by troglomorphic fish in the Northern Sierra de El Abra. The new population may be isolated from its nearest cave, Pachón cave, due to a fossil canyon. This new population corresponds to a mixed population of cavefish, surface fish, and intermediate individuals in morphology, due to intermittent contact with the Arroyo Lagartos surface population, as evidenced by the phenotypic and mitochondrial data. This discovery opens the possibility of describing the historic and recent events of cave colonization from their surface ancestors. But most important, it will allow for a better understanding of the evolutionary history of Pachón cave, and the Northern part of the Sierra de Abra.

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Supplementary material 1

DNA sequence of the mitochondrial 16S rRNA

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Data type: Nucleotide differences between lineages

Explanation note: El Refugio cavefish had identical sequences to fish from Pachón cave and from the local surface *Astyanax mexicanus* (Haplotype A). Compared to the mitochondrial haplotype B of cavefish from Sabinos, Tinaja, and Curva, they differed by 5 bp.

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Link: https://doi.org/10.3897/subtbiol.45.98434.suppl1

Supplementary material 2

Geometric morphometric coordinates, PCA values obtained from the geometric morphometric data and orbit diameter values for the populations

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Data type: Morphological data

Explanation note: SM2_T1. Geometric morphometric coordinates. SM2_T2. PCA values obtained from the geometric morphometric data. SM2_T3. Orbit diameter values for the populations.

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Link: https://doi.org/10.3897/subtbiol.45.98434.suppl2

Supplementary material 3

Contrasting vertical isolation between Pachón and El Refugio caves and the fossil canyon between Pachón and El Refugio caves

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Data type: Geographic data

Explanation note: **A**) Contrasting vertical isolation between Pachón and El Refugio caves. In blue are the temporal streams, source of surface fish. Arrows point downstream. The arrow's base is at the spring where the stream is born during the rainy season. Pachón is perched on a steep hill, so it is difficult to access by surface fish. Refugio cave on the contrary is at the same base level as the surface temporal stream and thus has easy access for surface fish to hybridize with the troglomorphic population. **B**) The fossil canyon between Pachón and El Refugio caves. The Río Comandante, used to ran through this fossil canyon but changed its course to the north, to its present position at La Servilleta canyon.

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